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ORIGINAL PAPER

Production of Strigolactones by *Arabidopsis thaliana* responsible for *Orobanche aegyptiaca* seed germination

Yaakov Goldwasser · Kaori Yoneyama · Xiaonan Xie · Koichi Yoneyama

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Abstract The germination stimulants produced by *Arabidopsis thaliana*, a host of root parasitic plants *Orobanche* spp. but not of arbuscular mycorrhizal (AM) fungi were examined. Root exudates from the hydroponically grown *A. thaliana* plants were subjected to reverse phase high performance liquid chromatography (HPLC) and retention times of germination stimulants inducing *O. aegyptiaca* seed germination were compared with those of strigolactone standards. In addition, the root exudates were analyzed by using HPLC linked with tandem mass spectrometry (LC/MS/MS). *A. thaliana* was found to exude at least three different germination stimulants of which one was identified as orobanchol. This is the first report of strigolactone production by a non-mycotrophic plant. These results together with recent knowledge imply that strigolactones have other unrevealed functions in plant growth and development.

Y. Goldwasser (✉)
R.H. Smith Institute of Plant Sciences & Genetics in Agriculture, Faculty of Agricultural, Food & Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel
e-mail: gold@agri.huji.ac.il

K. Yoneyama · X. Xie · K. Yoneyama
Weed Science Centre, Utsunomiya University, Utsunomiya 321-8505, Japan

Keywords Arbuscular mycorrhizal fungi · Brassicaceae · Germination stimulant · Orobanchol · Root parasitic plants

Abbreviations

DAP	Days after planting
EtOH	Ethanol
HPLC	High-performance liquid chromatography
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
MeOH	Methanol
MRM	Multiple reaction monitoring

Introduction

The seeds of the obligate root parasitic plants *Orobanche* spp. and *Striga* spp., now under the common botanical family Orobanchaceae, have unique requirements for germination. At first the seeds require a conditioning period of specific environmental conditions of high moisture and warm temperatures and then they require irreplaceable exposure to exogenous germination stimulants produced by plant roots (Parker and Riches 1993).

The known *Orobanche* seed germination stimulants belong to three chemical families: dihydrosorgoleone, sesquiterpene lactones, and strigolactones

(Bouwmeester et al. 2003). Among these germination stimulants, strigolactones have recently been shown to be distributed widely in the plant kingdom as host recognition signals for arbuscular mycorrhizal (AM) fungi from which plants benefit (Akiyama et al. 2005; Akiyama and Hayashi 2006; Bouwmeester et al. 2007).

Strigolactones are composed of a tricyclic lactone which connects via an enol ether bridge to a butyrolactone D ring. All naturally occurring strigolactones identified so far posses at least one methyl substituent on the A ring and various hydroxylation around the A and B rings (Humphrey and Beal 2006). Strigolactones are abundant in plants from a wide range of botanical families, hosts and non-hosts of root parasitic plants, and exhibit high *Orobanche* seed germination stimulation activity at extremely low concentrations.

To date, eight naturally occurring strigolactones have been identified in plant root exudates (Fig. 1). Strigol and strigyl acetate, the first strigolactones, were isolated by Cook et al. (1966, 1972) as *S. lutea* germination stimulants from the root exudates of cotton (*Gossypium hirsutum* L.), a non-host of *Striga* spp. Strigol was then identified by Siame et al. (1993) in the root exudates of the true *Striga* hosts, sorghum (*Sorghum bicolor*), maize (*Zea mays*) and proso millet (*Panicum miliaceum*). Sorgolactone was isolated by Hauck et al. (1992) from the root exudates of sorghum. Alectrol was isolated by Müller et al. (1992) as an *Alectra* and *S. gesnerioides* germination stimulant from the root exudates of cowpea (*Vigna unguiculata*) and has recently been identified as orobanchyl acetate (Matsuura et al. 2008; Xie et al. 2008). Orobanchol was discovered as the first *Orobanche* seed germination stimulant in the root exudates of red clover (*Trifolium pratense*), a host of *Orobanche minor* (Yokota et al. 1998). Solanacol, a tetradehydro-strigol isomer, and 2′-epi-orobanchol were isolated very recently as novel germination stimulants from the root exudates of tobacco (*Nicotiana tabacum*) by Xie et al. (2007). 5-Deoxystrigol was first discovered as an AM fungi branching factor in *Lotus Japanicus* root exudates (Akiyama et al. 2005) and then as one of the major germination stimulants in the root exudates of sorghum, maize and pearl millet (*Pennisetum typhoideum*) (Awad et al. 2006).

In addition to these known strigolactones, we have detected several novel strigolactones from tomato (*Lycopersicon esculentum*), pea (*Pisum sativum*), carrot (*Daucus carota*), tobacco (*Nicotiana tabacum*), Chinese milk vetch (*Astragalus sinicus*), eggplant (*Solanum melongena*), cucumber (*Cucumis sativus*), linseed (*Linum usitatissimum*) and sorghum (Awad et al. 2006; Yoneyama et al. 2006; Xie et al. 2007).

Arabidopsis thaliana belongs to the Brassicaceae family and is widely used as an amenable model plant system to study host-pathogen interactions including the elucidation of the germination factors for parasitic plants and the genetic factors governing their production (Westwood 2000; Goldwasser et al. 2002; Bouwmeester et al. 2003). Other Brassicaceae plants that have been reported as susceptible to *Orobanche* spp. include *B. oleracea*, *B. juncea* and *B. napus* (Foy et al. 1989). *A. thaliana* was found to induce seed germination of and was susceptible to *O. aegyptiaca*, *O. ramosa*, and *O. minor* (Goldwasser et al. 2000). In addition, low germination stimulation mutants of *A. thaliana* have been screened (Goldwasser and Yoder 2001). Elucidation of the factors regulating parasitic plants seed germination is important for understanding the initial steps of parasitism. This knowledge can be utilized to develop control measures against these crop devastating parasitic plants, based on manipulation of this process: inhibition of germination or induction of suicidal germination.

The objective of this study was to develop an experimental system to grow *A. thaliana* plants hydroponically, collect the exudates produced by their roots, and characterize the germination stimulants in the root exudates.

Materials and methods

Chemicals

(+)-Orobanchol and (+)-orobanchyl acetate were purified from red clover root exudates (Xie et al. 2007). (+)-Solanacol was obtained from tobacco root exudates (Xie et al. 2008). (+)-Strigol, (±)-sorgolactone, and (±)-5-deoxystrigol were generous gifts of Emeritus Prof. Kenji Mori (The University of Tokyo), Prof. Yukihiro Sugimoto (Kobe University) and Prof. Kohki Akiyama (Osaka Prefecture University), respectively. The synthetic strigolactone GR24 was kindly supplied by Prof. B. Zwanenburg (Radboud University, The Netherlands). The other

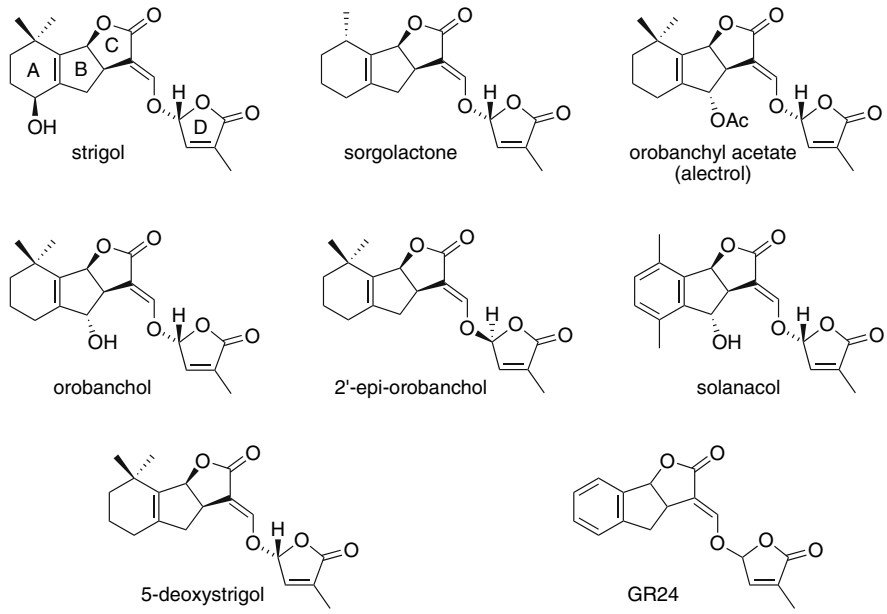

Fig. 1 Chemical structures of strigolactones

chemicals of analytical grade and HPLC solvents were obtained from Kanto Chemical Co. Ltd. and Wako Pure Chemical Industries Ltd.

A. thaliana hydroponic culture

Several systems were examined in order to find an efficient hydroponic culture system for growing *A. thaliana* plants and collecting their root exudates. Though many systems have been reported, we found most of them were not suitable for our specific purposes. The present study was based on a hydroponic culture system described by Toda et al. (1999).

The culture apparatus was made of a 50 mm × 50 mm slide frame mount in which a 40 × 20 mm 50 mesh nylon screen was inserted in the space intended for the 35 mm film. Wild type *A. thaliana* 'Colombia' seeds (Lehle Seeds) were surface sterilized with 70% EtOH for 10 min followed by treatment with 1% sodium hypochlorite with 0.1 ppm Tween 20 for 10 min. After washing with sterilized Milli-Q water, seeds were placed in the dark at 4°C for 48 h. The seeds were then suspended in sterilized Milli-Q water and pippetted onto the nylon screens placed in the slide frames, 40 seeds per slide. The slides were then placed floating in shallow 250 ml plastic trays (140 × 140 × 23 mm, $W \times L \times H$) containing 150 ml of 1/2 Tadano and Takano growth medium (Tadano and Tanaka 1980), 4 slides per tray. The trays were then placed in a growth chamber maintained at 23°C with a 16 h photoperiod at 100 μmole s^{-1} m^{-2}. Growth media were replaced every 3 days. Four weeks after sowing, media in the containers were substituted with tap water. The water in which roots grew in was replaced every 2 days for the following 10 days and replenished with fresh tap water. Every 2 days, the collected water containing the root exudates was placed in a flask to which 1 g of charcoal (activated carbon for column chromatography, Wako Pure Chemical Industries Ltd., Tokyo) was added and thoroughly mixed in order to absorb the stimulants.

Extraction of root exudates

Root exudates absorbed on the activated carbon were eluted with acetone, and the acetone was evaporated in vacuo. The residue was dissolved in 50 ml of 0.2 M K_2HPO_4 and extracted 3 times with 50 ml of EtOAc. After drying over anhydrous $MgSO_4$, the EtOAc extracts were concentrated in vacuo. These crude extracts were stored in sealed glass vials at 4°C until use.

Characterization of strigolactones

Characterization of strigolactones in the root exudates from *A. thaliana* grown hydroponically was conducted by comparing retention times of germination

stimulants on reverse phase HPLC with those of synthetic (or natural) standards and by using HPLC connected to tandem mass spectrometry (LC/MS/MS) (Sato et al. 2003, 2005; Awad et al. 2006).

HPLC separation

HPLC separation was performed with a U980 HPLC instrument (Jasco, Tokyo, Japan) fitted with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 mm, 5 µm, Kanto Chemicals Co. Ltd., Tokyo, Japan). Crude extracts were dissolved in 60% MeOH, filtered through spin columns (Ultra-Free MC, 0.45 µm pore size, Millipore), and 10 µl was injected. The initial mobile phase used was 60% MeOH in water and was changed to 100% MeOH 30 min after injection. The column was then washed with 100% MeOH for 20 min. The flow rate was 0.2 ml/min and the column temperature was set to 40°C.

Mass spectrometry

Mass spectrometry was conducted on a Quattro LC mass spectrometer (Micromass, Manchester, UK) with an electrospray source. Both the drying gas and the nebulizing gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). Nebulizer gas flow was set to 100 l h^{-1} and desolvation gas flow to 500 l h^{-1}. Interface temperature was set to 400°C and source temperature to 150°C. The capillary and cone voltages were adjusted to fit each molecule and to the ionization mode (positive and negative). The collision gas was argon, and the collision energy was optimized for each compound. The collision gas pressure was 0.15 Pa. Data acquisition and analysis were performed by MassLynx software (version 4.1).

O. aegyptiaca seed germination bioassays

O. aegyptiaca seeds were collected in the summer of 2002 from a heavily infested chickpea (*Cicer arietinum*) field in Gesher Haziv, Western Galilee, Israel. The dry *O. aegyptiaca* seeds were cleaned and then placed in a closed plastic container at room temperature until use.

The seeds were surface sterilized with 70% EtOH for 10 min followed by treatment with 1% sodium hypochlorite with 0.1 ppm Tween 20 for 20 min. After thorough washing with sterile Milli-Q water, the seeds were placed on a moist filter paper in a 9 cm Petri dish. The Petri dishes were sealed with parafilm, wrapped with aluminium foil and kept at 25°C for 1 week (conditioning). The conditioned seeds were suspended in sterilized Milli-Q water and 10–20 seeds were pipetted onto 6 mm glass fibre paper discs. Four inoculated discs were placed on a 5 cm filter paper disc lining a 5 cm Petri dish. Two hundred µl of root exudate samples or authentic strigolactones in MeOH were applied to each Petri dish and after evaporation of the MeOH, the 6 mm discs carrying the conditioned seeds were placed on the 5 cm filter paper and the filter paper was moistened with 600 µl sterile Milli-Q water. Petri dishes were sealed with parafilm, wrapped with aluminium foil and placed at 25°C for 5 days. The Petri dishes were then opened and seed germination percentage was determined under the stereoscopic microscope. Petri dishes with seeds treated with and without 10^{-6} M GR24 were always included as the positive and the negative controls. All treatments were replicated 3 times.

To examine distribution of germination stimulation activity after reverse phase HPLC separation, the eluant was directly collected with 5 cm Petri dishes lined with a filter paper. The fractions were collected every minute for 35 min after injection. The Petri dishes were then taken to the hood, opened to allow the solvent to evaporate, moistened with 600 µl sterile Milli-Q water and 6 mm glass fibre discs carrying conditioned *O. aegyptiaca* seeds were placed on the treated filter paper as described above.

Results and discussion

The *O. aegyptiaca* seed germination bioassay was first conducted with the combined crude extract to confirm the presence and to estimate relative concentration of stimulants. The combined extracts induced 1, 12, and 56% seed germination at concentrations of 1×10^{-5}, 1×10^{-4}, and 1×10^{-3}, respectively (Fig. 2). The synthetic strigol analogue GR24, the positive control, elicited 82% germination

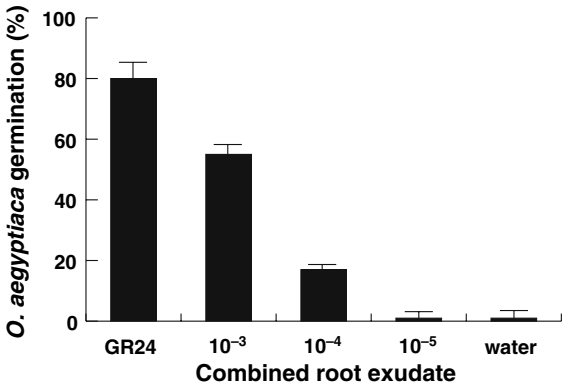

Fig. 2 Germination stimulation activity on *Orobanche aegyptiaca* seeds of combined root exudates from *Arabidopsis thaliana* grown in the slide frame hydroponics culture system. The combined root exudate was diluted with MeOH to concentrations of 1×10^{-3}, 1×10^{-4} and 1×10^{-5}. The synthetic strigol analogue GR24 (10^{-6} M) served as the positive control and water as the negative control. Data are means of 3 replications; vertical bars indicate the standard errors of the means

at 1×10^{-6} M while water, the negative control, induced 1% germination.

The time-course characteristic of seed germination activity of the crude extracts is shown in Fig. 3. The extracts at 1×10^{-3} induced 36 to 56% germination during the incubation period, germination rates that were similar to that elicited by the combined extracts mentioned above. At the 1×10^{-2} concentration the crude extracts from the first three sampling periods showed 79 to 89% germination that were similar to that achieved by 1×10^{-6} M GR24. These results indicate that during the sampling period of root exudates from mature *A. thaliana* plants with well-developed roots, 35 to 46 DAP, the production of *O. aegyptiaca* seed germination stimulants by *A. thaliana* roots is constant.

To detect known strigolactones by LC/MS/MS, we used 6-channel multiple reaction monitoring (MRM). The six channels were set to the 6 transitions of *m/z* $411 > 351$, $369 > 272$, $367 > 270$, $365 > 268$, $353 > 256$, and $339 > 242$ to detect strigyl acetate, strigol, didehydro-strigol, tetradehydro-strigol, 5-deoxystrigol, and sorgolactone, respectively.

In the 6-channel MRM chromatogram of *A. thaliana* root exudates, a distinct peak at the retention time of 8.2 min in the transition of *m/z* $369 > 272$ for monitoring strigol and its isomers was detected. This was identified as orobanchol by co-chromatography with pure orobanchol isolated from red clover root exudates (Fig. 4). There were distinct peaks at 4.4 min and 4.9 min in the MRM channels for didehydro- and tetradehydro-strigol isomers. Unfortunately, we could not identify these peaks because their retention times were different from those of a putative didehydro-strigol isomer and solanacol, the tetradehydro-strigol isomer, found in tobacco root exudates (Xie et al. 2007). No clear peaks were

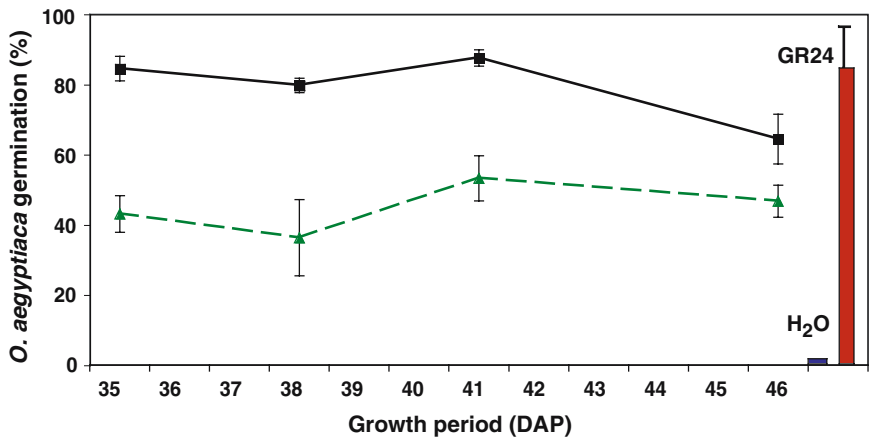

Fig. 3 Time-course characteristics of germination stimulation activity on *Orobanche aegyptiaca* seeds of root exudates from *Arabidopsis thaliana* grown in the slide frame hydroponics culture system. The root exudates were diluted in MeOH to concentrations of 1×10^{-2} (solid line) and 1×10^{-3} (broken line). GR24 (10^{-6} M) and water were used as the negative and positive controls, and germination rates are represented by bars. Data are means of 3 replications; vertical bars indicate the standard errors of the means

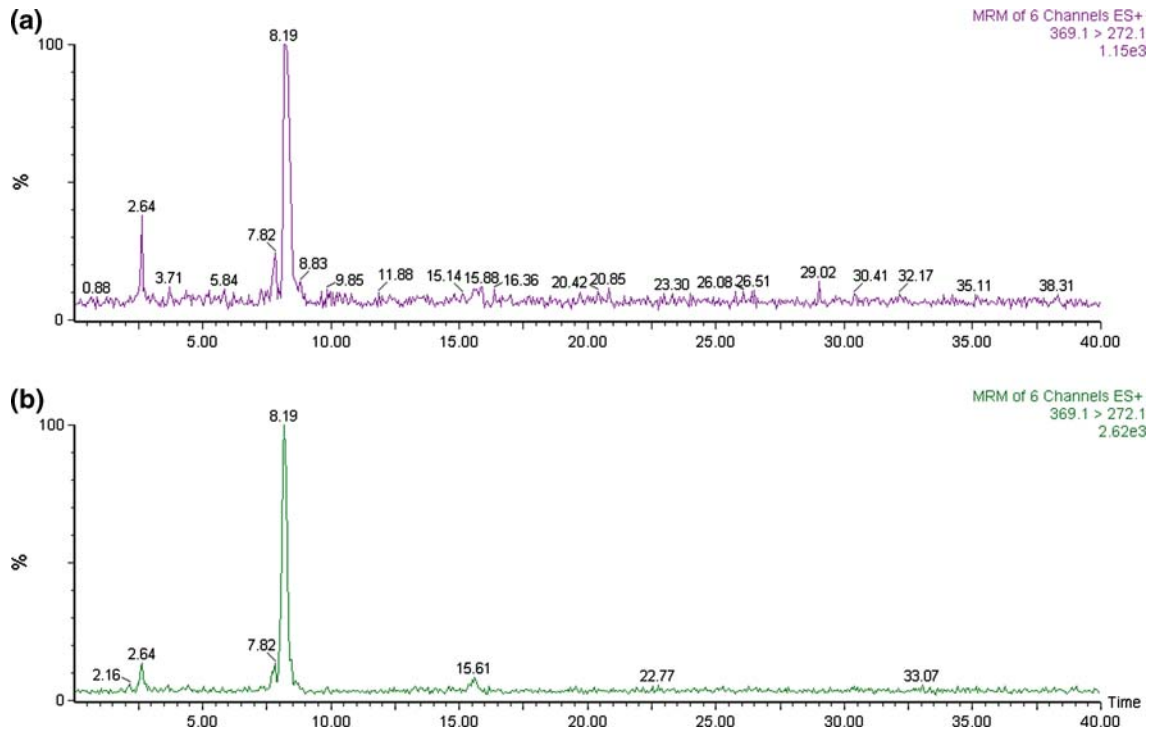

Fig. 4 Multiple reaction monitoring (MRM) chromatograms of *Arabidopsis* root exudate. (**a**) The MRM chromatogram, monitoring transition of *m/z* 369 > 272, of the crude extract. (**b**) The MRM chromatogram of the crude extract +50 nM orobanchol

detected in the channels for 5-deoxystrigol or sorgolactone, indicating that *A. thaliana* did not produce detectable amounts of these strigolactones under these experimental conditions.

A portion of the combined crude extract used for LC/MS/MS analysis was fractionated by reverse phase HPLC operated under the same conditions for LC/MS/MS analysis and the fractions collected every minute were examined for *O. aegyptiaca* seed germination stimulation (Fig. 5). For this, preliminary germination assays were conducted to determine an optimal dilution of the crude extract. In general, 100- to 1000-fold dilution of the samples which give small but distinct peaks of strigolactones in the MRM chromatogram was suitable for germination assays. When an excessive amount of crude extract was loaded to the HPLC, germination stimulation activity was distributed broadly in many fractions.

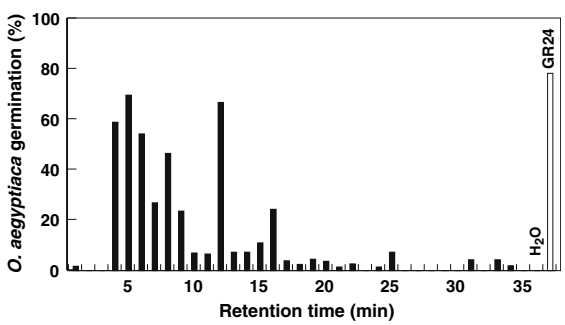

Fig. 5 Distribution of germination stimulation activity of *Arabidopsis* root exudate on *Orobanche aegyptiaca* seeds after reverse phase HPLC separation

The germination stimulation activity was detected in at least 4 fractions (eluted at 4–6 min, 7–9 min, 12 min, and 16 min) as shown in Fig. 5. The first active fraction eluted at 4–6 min may contain didehydro- and tetradehydro-strigol isomers. The second fraction eluted at 7–9 min was found to contain orobanchol as described before. The third and the forth fractions eluted at 12 and 16 min seem to have lipophilicities similar to that of strigyl acetate and orobanchyl acetate, respectively, but none of them were detected in the LC/MS/MS analysis.

Conclusion

In this study the strigolactones produced by *A. thaliana* roots that induce *O. aegyptiaca* seed germination were determined for the first time. We identified orobanchol as one of the major germination stimulants produced by *A. thaliana*., a host of *O. aegyptiaca* but not of arbuscular mycorrhizal fungi. In addition to orobanchol, *A. thaliana* was found to produce several germination stimulants probably including strigolactones like tetradehydro-strigol and didehydro-strigol isomers.

Orobanchol has been first identified in the root exudates of the *Orobanche* true host red clover (*Trifolium pratense*) (Yokota et al. 1998). Since then we have identified it as one of the major strigolactones in the root exudates of *Orobanche* hosts such as tobacco (Xie et al. 2007).

In this study we demonstrated for the first time that *A. thaliana*, a host of *O. aegyptiaca* but not of arbuscular mycorrhizal fungi produces strigolactones that act as *Orobanche* seed germination stimulants. The fact that strigolactones are abundant in nature in many botanical groups, host and non-hosts of root parasitic plants, and host and non-hosts of arbuscular mycorrhizal fungi, implies that these molecules may have other un-revealed significant roles in plants.

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